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Isolation and Identification of an Epibiotic Bacterium Associated with Heterocystous *Anabaena* Cells

BRADLEY S. STEVENSON[1,*] AND JOHN B. WATERBURY[2]

[1] *Department of Botany and Microbiology, University of Oklahoma, Norman, Oklahoma 73019; and*
[2] *Department of Biology, Woods Hole Oceanographic Institution, Woods Hole, Massachusetts 02543*

Heterotrophic bacteria are commonly found in close associations with photosynthetic cyanobacteria in aquatic ecosystems. Some of these associations can be species-specific and mutualistic, resulting in optimal growth and nitrogen-fixing potential for the cyanobacteria. A two-membered culture, consisting of a heterotrophic, epibiotic bacterium attached to an Anabaena *sp. was studied in the work reported here. The epibiotic bacterium was grown in pure culture, and both organisms were identified on the basis of their 16S rRNA gene sequence. The specificity of the epibiont for the* Anabaena *sp. heterocysts was confirmed by re-association experiments. The epibiont is a member of the Alphaproteobacteria in the order Rhizobiales, with close relatives that include a group of aerobic anoxygenic photosynthetic marine isolates commonly associated with dinoflagellate phytoplankton. The close association of the epibiotic bacterium with its* Anabaena *host, and its phylogenic affiliation allude to the evolutionary history of association with photosynthetic organisms for a group of* Rhizobia *and warrant further investigation.*

In diverse aquatic environments, bacteria routinely form close associations with photosynthetic cyanobacteria (1). These cyanobacteria represent a favorable habitat for heterotrophic bacteria because of their buoyancy, production of a mucilaginous sheath, and excretion of copious organic carbon and nitrogen compounds (2–4). Some epibiotic bacteria exhibit specificity for their cyanobacterial hosts through chemotaxis and specificity for the site of their attachment (5–7). These relationships are mutualistic: for example, epibiotic cells incorporate fixed organic carbon and nitrogen from the cyanobacterium *Anabaena* within an hour under optimal conditions (8). Aerobic respiration of the epibiont results in beneficial localized removal of O_2 and perhaps CO_2 recycling, which in turn leads to higher photosynthetic growth and N_2-fixing potential for the *Anabaena* (7, 9).

The work reported here focuses on a two-membered association between an epibiotic bacterium and the heterocystous cells of a species of *Anabaena.* This two-membered assemblage was isolated from a brackish marsh located in Woods Hole, Massachusetts, by capturing a single *Anabaena* filament with attached epibiotic cells, serially washing the filament in sterile autotrophic medium (25% SO) to remove any contaminating bacteria, and using the washed filament to inoculate an axenic culture of the *Anabaena* sp. (10). The resulting two-membered culture has been studied for at least 10 years as part of the curriculum of the Microbial Diversity summer course at the Marine Biological Laboratory in Woods Hole. The epibiotic bacterium was cultivated in the absence of its *Anabaena* host. Both bacteria that comprise this assemblage were characterized on the basis of the molecular phylogeny of their 16S rRNA gene sequences.

In cultures of the two-membered assemblage, 10 to 50 epibiotic cells were attached to each heterocyst (Fig. 1; panels A and B). Individual heterocysts severed from senescent *Anabaena* filaments were also observed to have numerous epibiont cells attached. The epibiont cells were 2 to 5 μm long, 0.2-μm wide gram-negative rods, narrower at one end. The thinner end of the cell was the site of attachment to the *Anabaena* heterocysts. Although the observation was not quantified, the epibiont cells attached preferentially to the polar ends of the heterocysts near the junction with the adjacent vegetative cells, as noted in studies of similar epibiont-*Anabaena* associations (7).

The epibiotic bacterium was successfully cultivated in the absence of the *Anabaena* basibiont in a brackish, heterotro-

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* To whom correspondence should be addressed. E-mail: bradley.stevenson@ou.edu

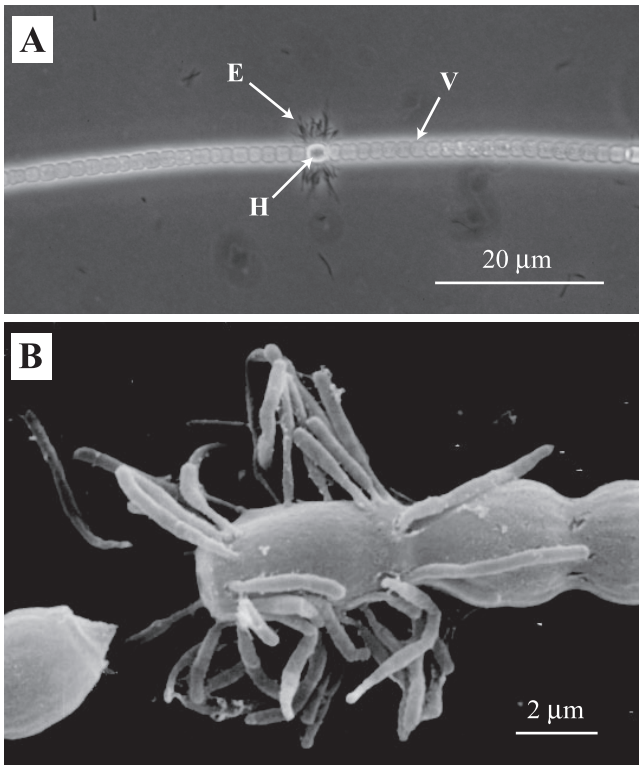

Figure 1. The two-membered culture growing in the brackish autotrophic medium ½ SO (10). (A) A filament of *Anabaena* sp. SSM-00, consisting of vegetative (V) and heterocystous (H) cells visualized using phase contrast microscopy. The epibiotic bacterial cells (E) can be seen attached exclusively to the heterocysts. (B) A scanning electron micrograph of a filament from the same culture, with epibiotic cells preferentially attached at the polar ends of the heterocyst near the junction between the heterocyst and vegetative cell. One of the adjacent vegetative cells has become detached from the heterocyst.

phic medium called Marine Purity (MP) medium (Fig. 2; panel A). An inoculum (1/100th volume) of the two-membered culture produced visible growth in MP medium after 7 days, and became turbid after 2 more days of vigorous shaking at room temperature. Cells clumped and settled to the bottom of the tube as the culture became turbid. The morphology of the cells in this heterotrophic culture closely resembled the epibiotic bacteria from the two-membered culture (Fig. 2; panel A). The cells exhibited a tumbling, clumsy motility, with many attached to one another at the narrow ends of the cell, forming rosettes and bundles. After at least three subsequent transfers to fresh MP medium (1/10,000th volume), no *Anabaena* cells could be detected microscopically, all cells shared the same morphology, and 16S rRNA gene analyses (discussed below) indicated that only one organism was present. Subsequently, a pure culture of the epibiont was established from spatially isolated colonies on 0.5× Marine Agar (MA) medium ([per liter] Difco Marine Agar 2216, 27.55 g; Difco Bacto Agar, 7.5 g) using the two-membered culture (in ½ SO medium (10)) as

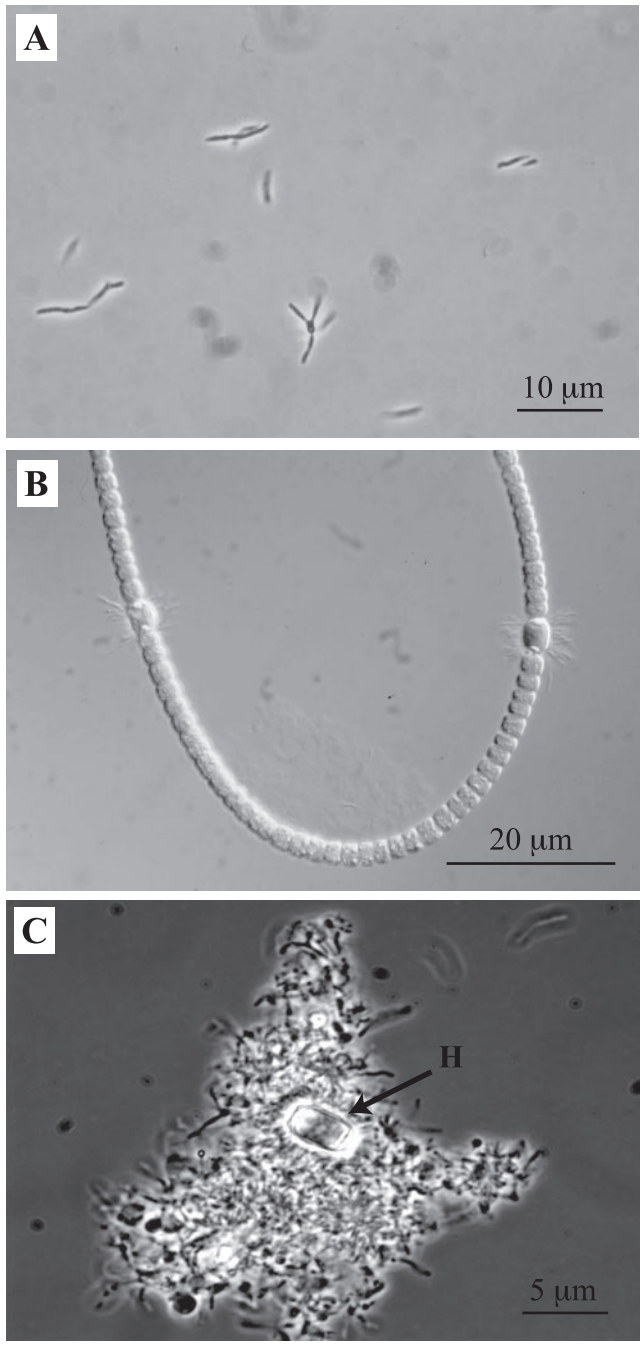

Figure 2. Heterotrophic enrichment of the epibiotic bacterium *Rhizobium* sp. WH2K, and a re-association experiment with epibiotic cells and an axenic *Anabaena* sp. SSM-00 culture. (A) A phase contrast photomicrograph of the epibiotic bacterium *Rhizobium* sp. WH2K growing in isolation from the *Anabaena* sp. in the brackish, heterotrophic marine purity (MP) medium ([per liter] NaCl, 20 g; AC Broth (Difco), 17 g; $MgSO_4$ $7H_20$, 8 g; $CaCl_2$ $2H_20$, 1.5 g.). The attachment of WH2K cells to heterocysts of an *Anabaena* sp. SSM-00 filament (panel B, DIC), and a detached heterocyst (H) (panel C, phase contrast) is shown, following a re-association experiment in which WH2K cells growing in MP medium were added to an axenic culture of the *Anabaena* sp. SSM-00.

the source of inoculum. The epibiont culture was considered pure after three successive transfers onto fresh MA medium. The purity of the culture and the identity of the epibiont were confirmed using microscopic analysis and 16S rRNA gene sequence. Growth of these cells in defined media, or any medium other than MP and MA, has been unsuccessful, severely limiting their metabolic characterization.

In a re-association experiment, cells from the heterotrophic culture were added to an axenic culture of the *Anabaena* sp. Within an hour, these cells could be seen attached to heterocysts in the same manner as in the two-membered culture (Fig. 2; B and C). Further indication that cells from the heterotrophic culture were the epibiotic bacterium came from an amplified ribosomal DNA restriction analysis (ARDRA) of cloned 16S rRNA genes from both cultures. Only two restriction patterns, or ribotypes, corresponding to the two bacteria in the assemblage, were obtained after screening more than 20 clones from the two-membered culture. One ribotype was obtained from the heterotrophic culture, and it matched one of those from the two-membered culture.

The identities and phylogenies of both the *Anabaena* sp. (SSM-00; NCBI accession no. DQ364237) and the epibiotic bacterium (*Rhizobium* sp. WH2K; NCBI accession no. DQ364238) were confirmed by sequence analysis of selected 16S rDNA clones. The nearest cultured relatives of the *Anabaena* sp. SSM-00 were other members of the Nostocales, *Anabaena cylindrica* and *Cylindrospermopsis raciborskii,* both sharing 96% sequence similarity with SSM-00 (Fig. 3). *Rhizobium* sp. WH2K was identified as a member of the phylum Alphaproteobacteria and the order Rhizobiales (Fig. 4). The closest relatives (97% sequence similarity) to WH2K included a marine heterotroph, *Hoeflea marina* LMG 128 (11), and several recently characterized epibionts associated with dinoflagellates (*e.g., Ahrensia* sp. DFL 44) (12).

WH2K and some of its close relatives share a common life history that includes close associations with photosynthetic organisms in marine or brackish environments (11, 12). The ability to fix N_2 is unknown for these organisms, but WH2K did not possess a dinitrogen reductase gene (*nifH*) that was amplifiable using gene-specific PCR primers (13). The dinoflagellate epibiont *Ahrensia* sp. DFL 44 contains the *pufLM* genes of the bacterial photosynthesis reaction center but did not produce bacteriochlorophyl *a* (Bchl *a*) under the conditions tested, and therefore is a putative aerobic, anoxygenic photosynthetic bacterium (12). WH2K was not screened for the *pufLM* genes, but like *Ahrensia* sp. DFL 44, it did not exhibit pigmentation suggestive of Bchl *a* in dark or light incubated cultures.

This work appears to be the first molecular analysis of an epibiont-*Anabaena* assemblage. No molecular taxonomic data seem to exist for other morphologically similar assemblages. Epibionts from other assemblages have been characterized, on the basis of morphological and physiological traits, as pseudomonads or *Zoogloea* sp. (5, 6, 9, 14, 15). The characterization of some of these epibionts as species of *Zoogloea* is problematic because, at the time, the genus was based solely on morphological and physiological traits and was later shown to incorrectly include members of both the *Alphaproteobacteria* and *Betaproteobacteria* (16). Although it is quite possible that these epibionts are closely related to *Rhizobium* sp. WH2K described in this study, the molecular taxonomic data to make this determination confidently are lacking.

Cultivation of *Rhizobium* sp. WH2K in isolation from its

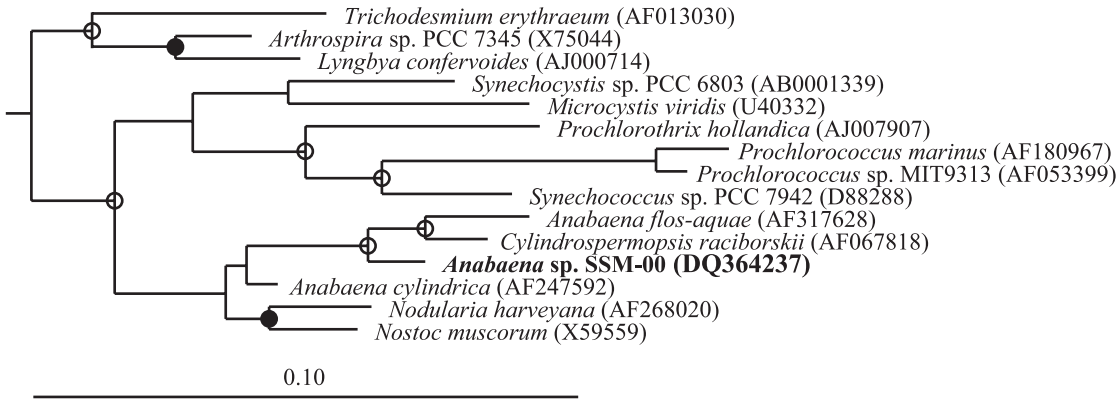

Figure 3. Maximum-likelihood tree showing the phylogenetic relationship between *Anabaena* sp. SSM-00 (bold) and representative cyanobacteria. NCBI accession numbers are given in parentheses. The nearly complete 16S rRNA gene was amplified with PCR using general bacterial primers (8F, 1492R), cloned, and sequenced (as in 17). This sequence was aligned with closely related representative sequences in a database using the ARB software package (18). Phylogenetic analyses were performed based on 1115 shared nucleotides using the maximum-likelihood algorithim within the ARB and PAUP 4.0b10 software packages (18, 19). Bootstrap values for branch points conserved in all analyses >75% (closed circles) and 50% to 75% (open circles) are indicated. The *Zea mays* chloroplast 16S rRNA gene sequence (Z00028) was used as an outgroup.

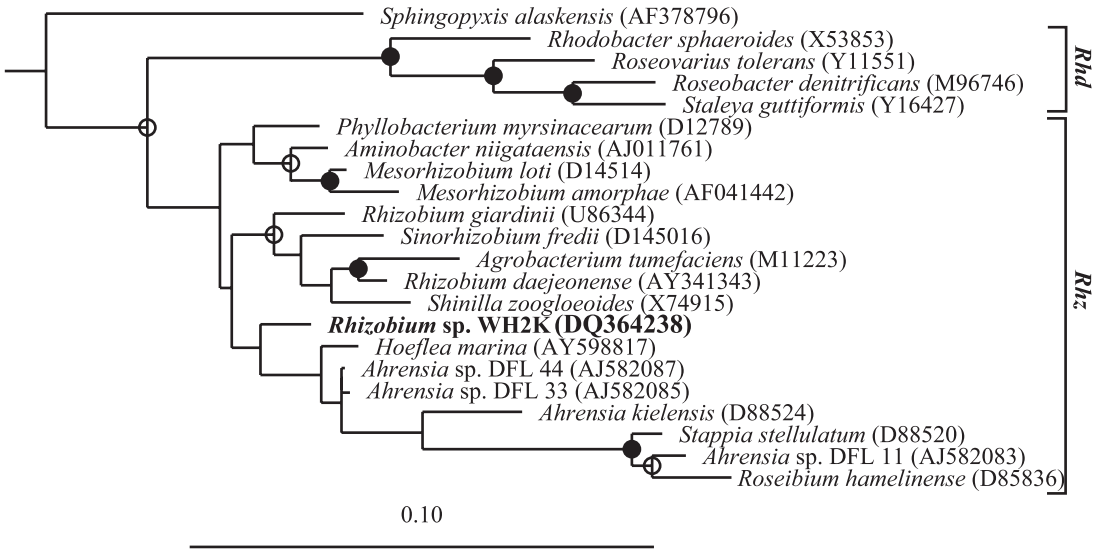

Figure 4. Maximum-likelihood tree showing the phylogenetic relationship between the epibiotic bacterium *Rhizobium* sp. WH2K (bold) and representative Alphaproteobacteria. NCBI accession numbers are given in parentheses. Brackets indicate the grouping of sequences within the orders Rhodobacterales (Rhd) and Rhizobiales (Rhz) inferred by phylogeny and the current hierarchy model in Bergey's Taxonomic Outline (May 2004 release: available online after free registration at http://dx.doi.org/10.1007/bergeysoutline). The nearly complete 16S rRNA gene sequence (1148 nt) was amplified, cloned, sequenced, aligned, and analyzed phylogenetically as described in Fig. 4. The Gammaproteobacterium *Allochromatium vinosum* (M26629) was used as an outgroup.

basibiont *Anabaena* sp. SSM-00 has opened the door to further investigate its close association with *Anabaena* sp. SSM-00, as well as to phenotypic and genotypic characterization. Phylogenetic characterization of the epibiotic bacterium *Rhizobium* sp. WH2K has revealed a group of *Rhizobia* that share a life history of close associations with photosynthetic organisms that include legumes, phytoplankton, and filamentous cyanobacteria. Additionally, this work provides a new perspective on the symbiotic relationship between epibiotic bacteria and *Anabaena* sp. that has been studied for over 20 years. Further study of these phylogenetically related epibiotic and epiphytic bacteria could shed new light onto the evolution and ecology of mutualistic relationships between heterotrophic bacteria and photosynthetic organisms.

Acknowledgments

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